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14. ABSTRACT

Loss of estrogen receptor (ER) function has been associated with hyperactive ERK1/2, which culminates in aggressive, radiation resistant cancers. The ERK1/2 pathway has also been linked to DNA damage and repair, with multiple proteins involved in DNA repair being transcriptionally regulated through ERK1/2-dependent signaling. An increased DNA repair capacity in ER-a negative breast tumors has been implicated as a mechanism of radioresistance. We postulate that the mechanism of development of radiation resistance in the ER-a negative breast cancer cells involves a dynamic interplay between the ERK1/2 pathway and DNA repair proteins. We compared ER-a positive and negative cells for expression levels of ERK1/2 and DNA repair proteins involved in the repair of radiation-induced double strand breaks. Preliminary data obtained from clonogenic cell survival assays showed that ER-a positive cells were more radiosensitive compared with the ER-a negative cells. These cell lines are also being compared for the expression of ERK1/2 and its downstream proteins and proteins involved in DNA repair by Western blot analysis. We are also evaluating the ability of inhibitors of the ERK1/2 pathway to restore radiosensitivity to the ER-a negative cell lines. The effect of these inhibitors on expression of DNA repair proteins and their ability to restore ER-a expression will also be tested. The outcome of these studies will have a potential impact in the clinic and benefit breast cancer patients

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Introduction

Breast cancer is the most commonly occurring cancer among women (22% of all cancers in 2000) and is second only to lung cancer as a cause of cancer deaths in women (15% of cancer deaths) (1, 2). The estimated annual incidence of breast cancer worldwide is about one million cases with ~200,000 cases in United States (27% of all cancers in women) and ~320,000 cases in Europe (31% of all cancers in women) (3, 4). Over the last two decades, the annual incidence rate in US has been increasing steadily (5). Women with an early diagnosis and favorable risk factors are cured by primary surgical and radiotherapy treatment while those with more advanced or aggressive tumors experience recurrence and later death (5). Risk factors for recurrence are generally related directly or indirectly to the rate of cell proliferation and the percentage of cells undergoing apoptosis. The factors controlling these two interrelated processes are complex and not fully understood.

Radiotherapy of patients with breast cancer remains an important cancer treatment modality and plays an essential role in local and regional control of the disease (6). It has been estimated that more than 50% of all cancer patients receive radiation as part of their overall management. Randomized trials have demonstrated the efficacy of radiation therapy in the treatment of breast cancer. Even though many of these patients benefit from their treatment, between 30-50% of patients with localized disease initially fail at their primary tumor sites following therapy. A variety of strategies have been and are continuing to be actively explored to improve local control. Tumors locally fail after radiation therapy due to biological factors associated with the particular tumor. Advances in our knowledge of the molecular pathways that govern some of these factors has generated many new ideas that can be explored for improving the efficacy of radiation therapy but there are still aspects of tumor sensitivity to radiation that are poorly understood (7-9).

Since radiation therapy plays a critical role in the management of a majority of breast cancer patients, identification of factors that help predict which patients are at risk for relapse within the irradiated field remains an active area of investigation. A substantial amount of research has been devoted to identifying predictive markers for radiation resistance. Loss of estrogen receptor (ER) function has been associated with constitutive and hyperactive MAPK (particularly ERK1/2), which culminates in aggressive, metastatic, radiation-resistant cancers. Activation of the ERK1/2 cascade modulates the phosphorylation and activity of several nuclear transcription factors that in turn regulate a series of genes involved in promoting cellular survival and resistance to chemotherapy and ionizing radiation. The ERK1/2 pathway has also been linked to DNA damage and DNA repair, with multiple proteins involved in DNA repair being transcriptionally regulated through ERK1/2-dependent signaling (10-21). An important hallmark that dictates the radioresistant phenotype of tumor cells and is probably the most critical factor in the radiation responsiveness of a tumor is the ability of a cancer cell to repair and recover from radiation-induced DNA double-strand breaks (DSBs). An increased DNA repair capacity in ER-α negative breast tumors has also been implicated as a mechanism of radioresistance. We postulate that the mechanism of development of radiation resistance in the ER-α negative breast cancer cells involves a dynamic interplay between the ERK1/2 pathway and DNA repair proteins.

Body:

Breast cancer is a heterogeneous disease, displaying wide variances in response to various therapeutic approaches and outcome. Generally, hormone receptor negative tumors are high grade, poorly differentiated tumors. In accordance with these observations, decreased survival rates are reported for patients with estrogen- or progesterone-receptor negative tumors compared to those with hormone receptor positive breast cancer (22, 23).

The epidermal growth factor receptor (EGFR)/Her-2/neu/Ras/MEK/mitogen activated protein kinase (MAPK) and the c-kit-Akt / PI3K (phosphoinositol-3-kinase) pathways are two major signal transduction pathways that lead to activation of intracellular driving mechanisms for proliferation and antiapoptotic features of tumor cells. It has been previously demonstrated that MAPK family members, including ERK, JNK and p38 MAPK play an active role in the proliferation, invasive capacity and generation of metastatic potential for cancer cells, as well as chemoresistance (10-21). Furthermore, the MAPK family has been shown to have a regulatory role in providing the complex balance between cellular growth and death through competing

interactions. Therefore, the exact mechanism by which MAPK is involved in the pathogenesis of breast cancer is not clear and remains to be elucidated further.

Intracellular signaling through the Ras-MAPK pathway has been observed in a wide range of breast tumors and has been linked to non-genomic estrogen-mediated tumor growth and induction of estrogen receptor-negative phenotype, in addition to resistance to hormonal agents, such as tamoxifen (24-33). MAPK overexpression has also been associated with growth factor related and anchorage-independent tumor proliferation by increased heat shock protein expression in triple negative tumors and is in concordance with in vitro data suggesting that active MAPK signaling is correlated with estrogen receptor negativity and induction of receptor negative phenotype (24-33). The role of MAPK has not been extensively evaluated in a prospective trial, and data available is generally limited to analysis of archival material.

We postulate that the mechanism of development of radiation resistance in the ER- α negative breast cancer cells involves a dynamic interplay between the ERK1/2 pathway and DNA repair proteins.

Aim 2: Determine combinations of targeted therapeutics that will effectively restore sensitivity to ionizing radiation.

i). We will evaluate the ability of small molecule inhibitors (CI-1033 and UO126 to inhibit phosphorylated ERK1/2) to block constitutively activated ERK and restore radiosensitivity to the cell lines mentioned in Aim 1. Radiosensitization, determined on the basis of clonogenic survival, will be the critical endpoint of this series of experiments. (Months 14-20).

ii). We will test the effect of these inhibitors on inhibition of activated ERK, expression of DNA repair proteins and their ability to restore ER-α expression suggesting the presence of a feedback mechanism in modulating ER-α expression. We will use Western blot analysis to examine relative protein levels prior to and after treatment with these inhibitors. Any changes in the DNA repair capacity following treatment with the inhibitors will be assessed using the Comet and the Host cell reactivation assays. (Months 18-24).

ii). Construct MB231 cells (ER- α positive), stably transfected with an expression vector carrying activated ERK1/2 under an inducible promoter to serve as a model system to address the role of ERK1/2 in mediating a loss of ER-α expression and leading to radioresistance.

iii). Use siRNA approach to downregulate ER-α in MCF-7 cells and associate loss of ER- α to hyperactivation of ERK1/2 and DNA repair proteins.

iv). Carry out Host Cell Reactivation and Comet Assays to determine the intrinsic DNA repair capacity and the capacity to repair radiation-induced DNA double strand breaks in the cell lines mentioned above.

iv). Set up clonogenic cell survival assays to assess radiosensitivity of the above mentioned cell lines.

Aim 3: Generation of tissue arrays and immunohistochemical analysis of patient specimens for expression of DNA repair proteins and signaling intermediates in the ERK pathway.

i). We will evaluate the prevalence of the ERK pathway and its downstream targets, as well as DNA repair proteins (BRCA1, BRCA2, DNA-PK, GADD-45 and Topo-II α) in a cohort of clinical breast cancer specimens previously used to investigate for markers of locoregional failure after radiation therapy. An attempt will be made to correlate loss of ER-α with hyperactive ERK1/2 and high levels of DNA repair proteins in clinical samples. The samples will be analyzed by tissue microarray. (Months 24-36).

Key Research Accomplishments

The progress made towards each sub-specific aim is briefly summarized in this section.

In our previous report we had compared the intrinsic radiosensitivity of a panel of human breast cancer cell lines and shown that cell lines expressing estrogen receptor (MCF-7) were more sensitive to increasing doses of radiation when compared with the ER negative cells (MDA-MB-231, MDA-MB-453, MDA-MB-435 and Hs578t). ER- α negative cell lines had higher SF2 values when compared with the ER-α positive MCF-7

cells indicating intrinsic radioresistance of ER-α negative cells. In addition we tested MDA-MB-231 cells that were stably transfected with full length ER-α (clones designated ERα-3 and ERα-6). MB231 cells transfected with vector backbone were used as controls (designated LxSN2 and LxSN23). The estrogen receptor expressing ERα-6 clone was more sensitive to increasing doses of radiation when compared with the vector control cells. The survival enhancement ratio was enhanced when the estrogen receptor gene was put back into the cells. Both the cell lines were also compared for the level of expression of ER- α by western blot analysis. Following these experiments, we compared the basal levels of activated ERK1/2 and levels of DNA repair proteins (NBS1, RAD51, and Topo-IIα) in ER-α negative (MDA-MB-231, MDA-MB-468, MDA-MB-435 and Hs578t) and ER- α positive (MCF-7 and ZR75-1) breast cancer cell lines by Western Blot Analysis. ER- α negative cells had higher levels of phosphorylated ERK and DNA repair proteins such as phospho-NBS1 and RAD51. Levels of Topo-II α were also higher in ER- α negative breast cancer cell lines. However ZR75-1, an ER-α positive cell line, also expressed high levels of Topo-II α.

Since transient/constitutive expression of MAPK leads to downregulation of ER- α we obtained an MCF-7 breast cancer clone engineered to overexpress EGFR and thereby activated phospho-MAPK/ERK. This cell line, designated as MCE-5, was obtained from Dr. Dorraya El-Ashry (University of Michigan, Ann Arbor, MI). We compared the levels of pERK and ER- α in MCE-5 and MDA-MB-231 cells. The MCE-5 cells had a higher constitutive level of pERK when compared to MCF-7 cells. Exposure to 5Gy dose of radiation led to an increase in ERK levels in the MCF-7 cells but not in the MCE-5 or the MDA-MB-231 cells. Immunohistochemical analysis was also performed on the Hs578t, MDA-MB-231 and the MCE-5 cells for activated ERK. MDA-MB-231 and Hs578t cells showed positive staining for ERK. The MCE-5 cells overexpressing activated ERK however were very strongly positive for ERK by immunohistochemistry.

Since ERK is constitutively active in Hs578t and MDA-MB-468 cells as detected based on phospho-p44/p42 expression, we used the ERK inhibitor U0126 to test whether the MEK/ERK pathway was playing a role in radiation resistance of these ER- α negative cells. Treatment of Hs578t cells with 10μM dose of U0126 down-regulated pERK and this inhibition was sustained for up to 24 hrs of treatment (Figure 1A). To determine the ability of U0126 to act as a radiosensitizer, Hs578t cells were exposed to 10 μM U0126 for 24 hrs, irradiated and harvested for clonogenic assay. U0126 restored radiation sensitivity to Hs578t, ER- α negative cells, which are known to be extremely radioresistant (Figure 1B).

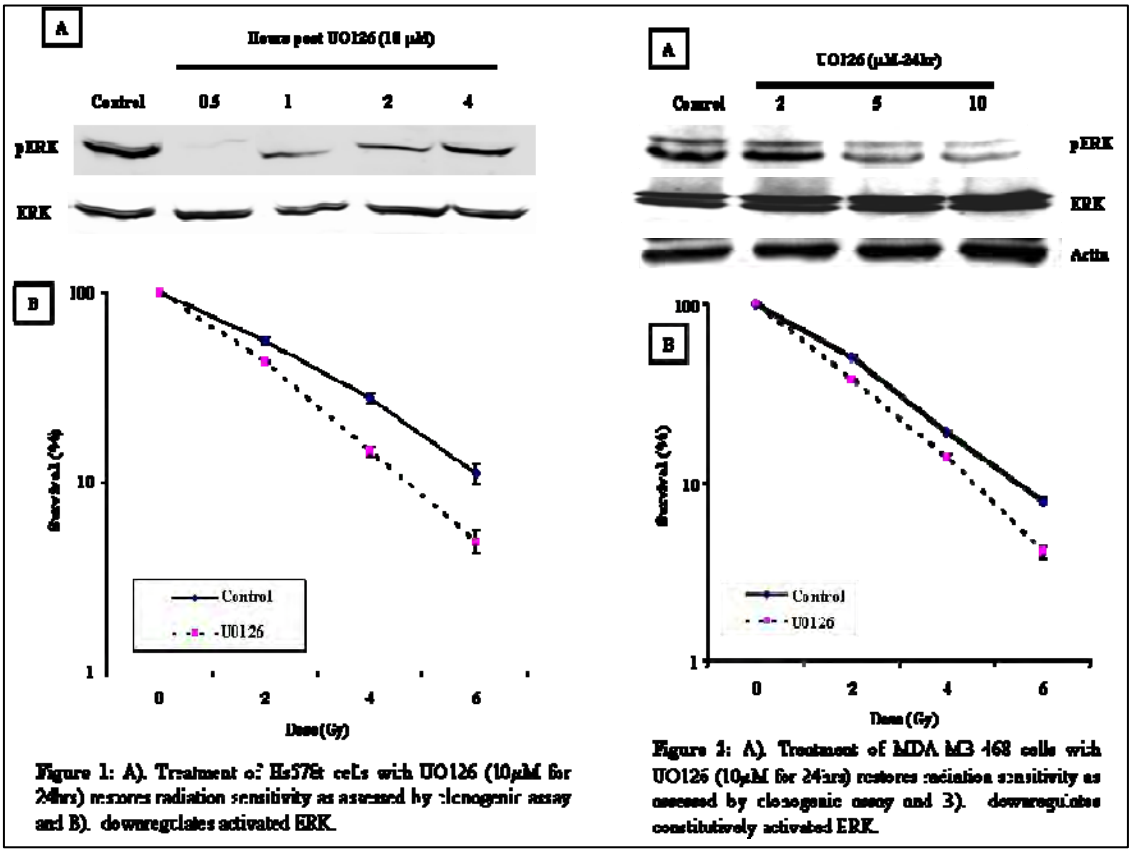

Figure 1: A). Treatment of Hs578t cells with U0126 (10μM for 24hrs) restores radiation sensitivity as assessed by clonogenic assay and B). downregulates activated ERK.

Figure 1: A). Treatment of MDA MB 468 cells with U0126 (10μM for 24hrs) restores radiation sensitivity as assessed by clonogenic assay and B). downregulates constitutively activated ERK.

Similar results were obtained with MDA-MB-468 cells (Figure 2A and 2B). Cells were treated with varying doses of U0126- 2, 5 and 10 μM for a period of 24 hrs. Treatment with both 5 μM and 10 μM dose of

U0126 suppressed phosphorylated ERK1/2 levels. Treatment with 10μM dose of U0126 also restored radiation sensitivity as assessed by clonogenic cell survival experiments.

At this point it was important to see if U0126 can restore radiation sensitivity following short treatments such as 30 minutes. MDA-MB-468 cells were treated with 10 μM dose of U0126- for a period ranging from 30 minutes to 24 hrs. Treatment with 10μM dose of U0126 suppressed phosphorylated ERK1/2 level as early as 30 minutes and also restored radiation sensitivity. For clonogenic cell survival experiments the cells were treated with 10μM U0126 for 30 minutes. This treatment was sufficient to restore sensitivity to radiation in the MB468 cells (Figure 3).

The next question we asked was whether MCF-7 cells which do not constitutively express ERK can be radiosensitized by U0126. To answer this, MCF-7 cells were treated with 10 μM U0126 for 24 hours and assessed for radiation response by clonogenic cell survival. U0126 treatment had a radioprotective effect on the MCF-7 cells (Figure 4) indicating that the ERK pathway does not mediate the radiation sensitivity of these cells.

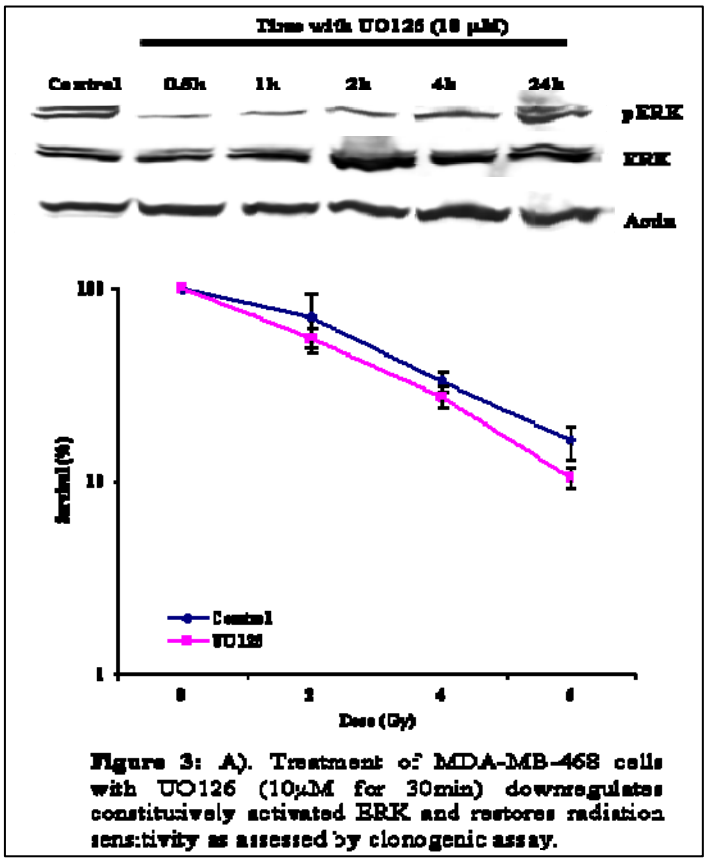

Figure 3: A). Treatment of MDA-MB-468 cells with U0126 (10μM for 30min) downregulates constitutively activated ERK and restores radiation sensitivity as assessed by clonogenic assay.

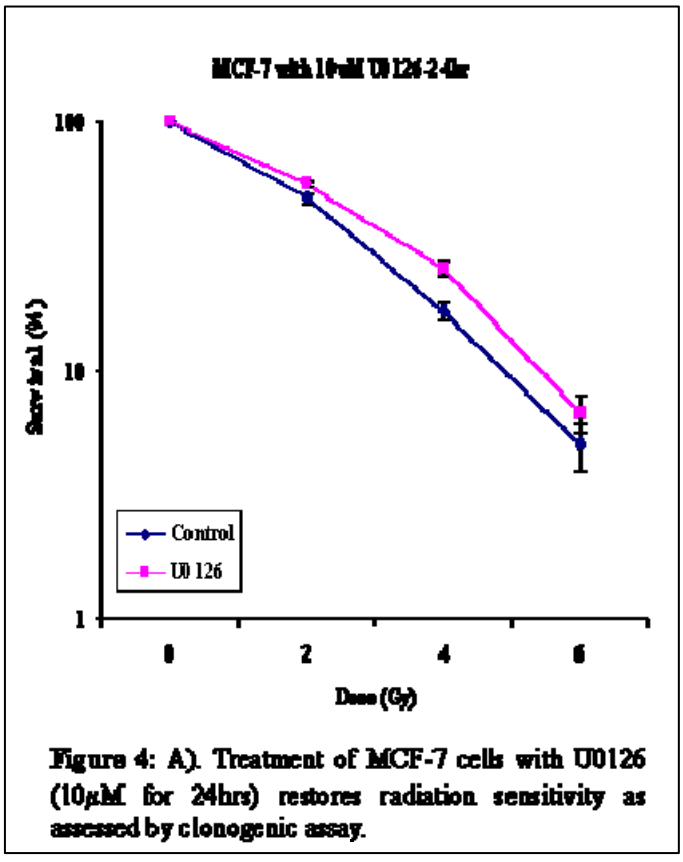

Figure 4: A). Treatment of MCF-7 cells with U0126 (10μM for 24hrs) restores radiation sensitivity as assessed by clonogenic assay.

To further test the loss of ER-α with radiation resistance we prepared an MCF-7 cell line in which ER-α levels were knocked down using shRNA to ER-α. MCF-7 cells which are ER-α positive, were stably transfected with shRNA to ER-α and several clones showing downregulation of ER-α were selected and analyzed by western blot analysis (Figure 5). These clones were further expanded and used for examine their sensitivity to radiation. We picked clone #4c for all further experiments as it showed good knockdown of ER-α when compared with the control MCF-7 cells and some of the other clones. Clonogenic cell survival experiments were set up to compare the radiation response of MCF-7 sh-Control cells versus MCF-7 sh-ER-α clone 4c.

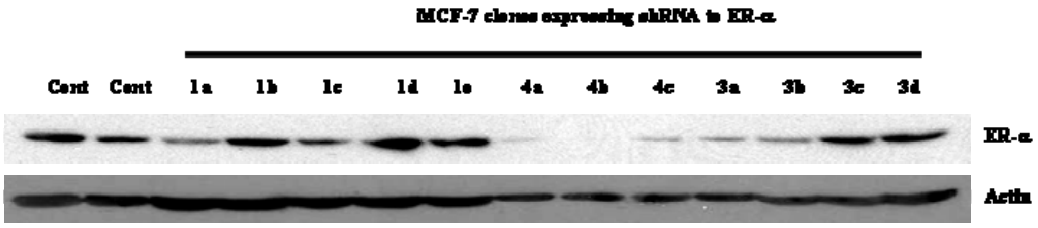

Figure 5: shRNA to ER-α was used to downregulate ER-α in MCF-7 cells and associate loss of ER-α to hyperactivation of ERK1/2 and DNA repair proteins

Knockdown of ER-α made the cells resistant to radiation as can be seen in Figure 6. Additionally, we saw a nice upregulation of ERK in these cells and believe that activated ERK might contribute to radiation resistance in these cells.

As an additional test of our hypothesis we have prepared MDA-MB-231 stable clones in which we have used a shRNA to knockdown expression of activated ERK1/2. These stable clones were characterized for downregulation of pERK1 or pERK2 by western blot analysis and then tested for their response to radiation. MDA-MB-231 clone with ERK1 knockdown was more sensitive to radiation when compared to the control transfected cells. The degree of sensitization was less than what we have obtained with U0126 but that could be attributed to the fact that U0126 downregulates both ERK1 and ERK2 whereas in the shRNA clone we are knocking down either ERK1 or ERK2 (Figure 7).

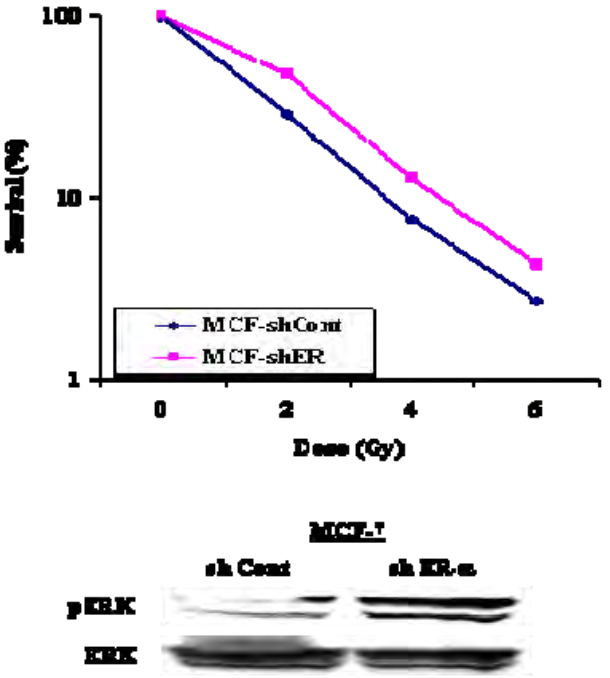

Figure 6: A). Knockdown of ER-α in MCF-7 cells radioprotects them and (B). leads to upregulation of pERK as seen by western blot analysis

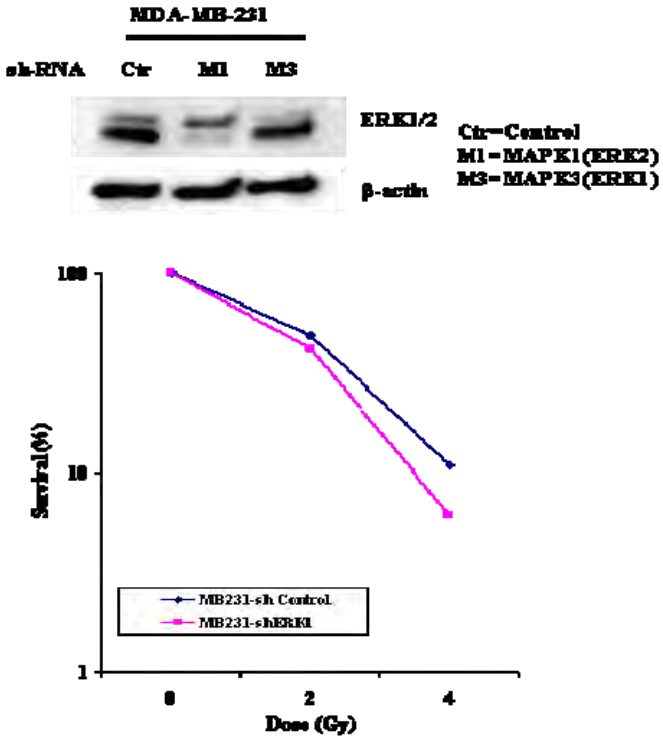

Figure 7: shRNA to ERK1 and ERK2 was used to downregulate ERK in MDA-MB-231 cells and associate loss of ERK1/2 to radiation sensitivity.

Since a lot of time was spent in preparing these stable clones we have just started analyzing these clones for their DNA repair capacity. However to make up for the time lost we started standardizing the immunohistochemistry procedure that we will be using extensively in the third year. We have already standardized the staining protocol for most of the antibodies that we will be using, this includes antibodies to DNA repair proteins as well as the ERK pathway.

Our preliminary IHC done on 3 different cells lines with varying estrogen receptor status (MDA-MB-231 : ER negative; MCF-7 shER: with estrogen receptor knockdown; and MCF-7 : estrogen receptor positive) demonstrates an abundance of most DNA repair proteins in the ER-negative cells (Figure 8 and 9). This is preliminary data and a detailed analysis requires to be done on these. We will have to examine in detail the subcellular distribution pattern of these proteins as well. In addition we have carried out IHC on commercially available tissue arrays in which the receptor status of the tissue samples is known (data not shown).

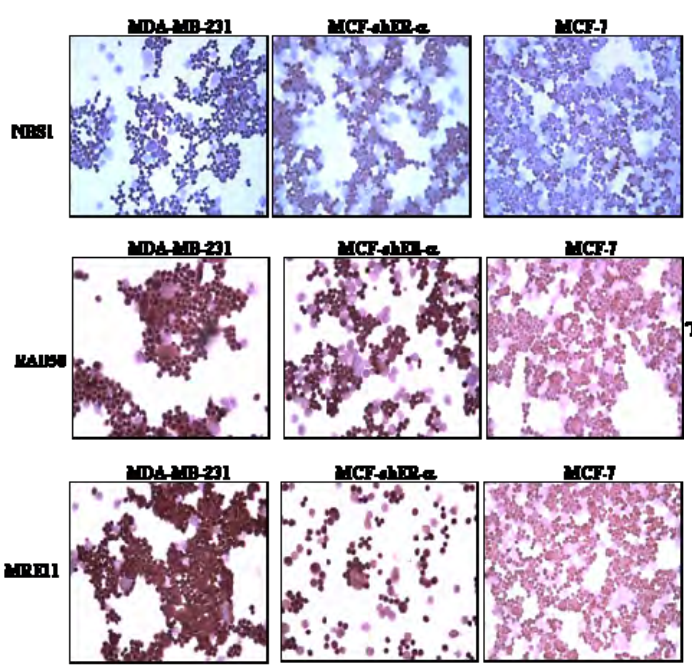

Figure 8: Immunocytochemistry on a panel of breast cancer cells with varying ER-α status.

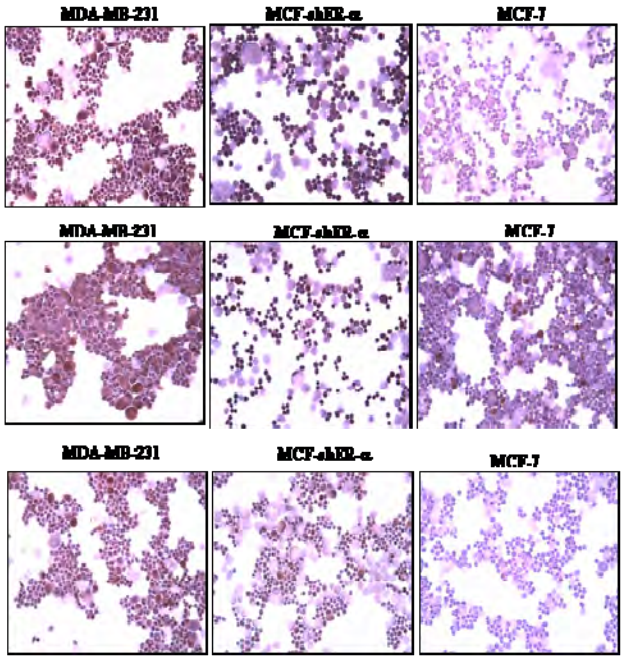

Figure 9: Immunocytochemistry on a panel of breast cancer cells with varying ER-α status.

Reportable Outcome: None

Conclusions: The project is currently progressing as planned and is in keeping with the timeline proposed.

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